

Organization and Transfer of Heterologous Chloramphenicol and Tetracycline Resistance Genes in *Pneumococcus*

NADJA B. SHOEMAKER, MICHAEL D. SMITH, AND WALTER R. GUILD*

Department of Biochemistry, Duke University, Durham, North Carolina 27710

Received for publication 5 February 1979

The *cat* and *tet* genes of chloramphenicol- and tetracycline-resistant clinical isolates of *Streptococcus pneumoniae* from Paris and Japan were shown to be contained in adjacent heterologous insertions into the chromosome. The two insertions transformed laboratory strains at frequencies that were low, unequal, and, for *tet*, very sensitive to the length of the donor deoxyribonucleic acid strand. In contrast, the transforming activity of *cat* was relatively stable. There was an unusual asymmetric cotransfer, in that a majority of the *tet* transformants also acquired *cat*, whereas only a few of the *cat* transformants also acquired *tet*. The evidence for chromosomal insertion came from genetic data showing linkage of *cat* to a chromosomal gene and from cosedimentation of *cat* with chromosomal markers in both velocity and dye-buoyancy experiments. Genes on a known plasmid introduced into pneumococcus from *Streptococcus faecalis* showed very different physical behavior. Most of the transformation properties of these genes can be readily accounted for by analogy to transformation of deletions of normal genes. Whether transposition contributes any of the transfers remains to be determined. The presence of one of the genes in the recipient promoted the integration of the other, demonstrating enhanced accumulation of heterologous genes by a process that did not involve plasmids in the species of concern.

The possibility that plasmid resistance factors are beginning to appear in pneumococcus (*Streptococcus pneumoniae*) has been raised by the increasing incidence of clinical isolates resistant to anywhere from 2 to 12 antibiotics (2, 8, 15, 19). Strains isolated in Niigata (19) and Paris (8) that were resistant to both chloramphenicol (Cm^r) and tetracycline (Tc^r) were each found to contain an inducible chloramphenicol acetyltransferase like that often associated with plasmids. Dang-Van et al. (8) found further (i) that on growth in ethidium bromide (EtBr) the Paris strain BM6001 segregated chloramphenicol-sensitive (Cm^s) variants at a frequency approaching 10⁻³, but these remained Tc^r, and (ii) that DNA from BM6001 transformed laboratory strains to chloramphenicol resistance only at low frequency and not at all to tetracycline resistance (<10⁻⁷/colony-forming unit). Although some of these properties suggested the presence of one or more plasmids, none was detected by DNA centrifugation methods (8).

We also could detect no plasmid in BM6001 by gel electrophoresis under conditions that readily showed pDP1, a cryptic 2.0-megadalton plasmid in another virulent encapsulated strain of pneumococcus (32). Although inconclusive, this result suggested that the resistance genes might be integrated into the chromosome. We

present below several lines of direct evidence that this is the case for the Paris strain and its derivatives. We have, however, constructed a pneumococcal strain carrying a plasmid transformed in from *Streptococcus faecalis* as a control to help demonstrate that the chloramphenicol and tetracycline resistance genes in the Paris strain are not plasmid associated. Because the results show that these genes are present as parts of nonhomologous insertions, they could represent transposons or remnants of such elements, but it would be premature to conclude that active transposition plays a role in their transformation. The Niigata strain (19) appears to be similar to BM6001 with respect to the organization and the locus of the chloramphenicol and tetracycline resistance determinants.

MATERIALS AND METHODS

Bacteria, growth, and transformation. Table 1 describes the pneumococcal strains used and their origins. Media, growth, preparation of competent cells, and exposure to DNA were as described previously (11). *ery-2*, *str-1*, and *nov-1* are chromosomal point mutations leading to resistance to erythromycin (Ery^r), streptomycin (Str^r), and novobiocin (Nov^r), respectively (11); *thy-1* is a spontaneous point mutation leading to a thymidine requirement (Thy⁻) and resistance to amethopterin; *cat* specifies chloramphenicol acetyltransferase, leading to chloramphenicol re-

sistance; and *tet* leads to tetracycline resistance.

Strain DP3200 was constructed by exposing competent Rx1 cells to a lysate of a strain of *S. faecalis* (constructed by V. Hershfield) that carries pIP501, a 20-megadalton plasmid carrying *cat* and *erm* genes that confer resistances to chloramphenicol and to the macrolide, lincosamide, and streptogramin B (MLS) group of antibiotics (13, 14, 33), which includes erythromycin. Transformants to erythromycin resistance and chloramphenicol resistance were selected, and all those tested were found to carry both. They also contained a plasmid that by electron microscopy and by migration in gels was near the size of pIP501, although identity has not yet been proven. DP3200 is one such transformant.

Donor DNAs. Crude lysates of the drug-resistant strains were prepared from 10- to 50-ml volumes of cultures grown to about 5×10^8 cells per ml by procedures that have been described previously (30), except that the lysates were stored at 4°C rather than frozen. Unless otherwise described, the lysates were one-fifth the original culture volume and contained 6 to 10 µg of DNA per ml. For transformation, 0.1 ml was used per ml of competent cells.

Shear treatments were by blending 3 ml in a 20-mm-diameter culture tube for 2 min on a laboratory mixer (Vortex, Jr.) or by passing 3 ml through a 3.8-cm no. 27 needle, attached to a 5-ml glass Luerlok syringe, five times at 0.4 to 0.5 ml/s.

Scoring of transformants. Scoring for drug resistance was by dilution and plating in an agar layer on 10-cm dishes, followed by an overlay of 8 ml of agar and 60 min of incubation for phenotypic expression and the addition of another 8 ml of agar with one or two drugs (15 µg of chloramphenicol, 5 µg of tetracycline, 0.5 µg of erythromycin, 200 µg of streptomycin, or 10 µg of novobiocin per ml). To score drug-sensitive transformants, we diluted the culture eightfold after DNase addition and incubated it for 3 to 4 h at 37°C to allow segregation before dilution and plating without drug. On the next day, colonies were transferred with sterile toothpicks to blood agar plates with and without appropriate drugs (5 µg of chloramphenicol, 2 µg of tetracycline per ml). Cotransfer of *cat* and *tet* was measured by toothpick transfer of Cm^r or Tc^r colonies to the other drug or by overlaying the cells with both drugs. The protocol for scoring *thy*⁺ transformants will be described elsewhere (J. E. Wagstaff, N. B. Shoemaker, and W. R. Guild, manuscript in preparation).

Centrifuge experiments. All runs were in 5-ml polyallomer tubes in an SW39 or SW50.1 rotor at 20°C. Sucrose gradients were as described in the legend to Fig. 1. For dye-CsCl gradients, cell lysates were mixed gently with CsCl and EtBr to a density of 1.56 g/ml and 70 µg of EtBr per ml (28) and centrifuged for 60 h at 33,000 rpm. Two-drop fractions were collected in dim light, and the EtBr was removed on small Dowex-50 columns (28). One-twentieth of each fraction was used for transformation.

RESULTS

In the initial experiment a gently prepared fresh lysate of BM6001 donated both *cat* and *tet*

TABLE 1. *Pneumococcal strains*

Strain	Relevant genotype ^a	Origin/reference
Clinical isolates ^b		
BM6001	<i>cat tet</i> (serotype 19)	8
N77	<i>cat tet</i>	19
Rx derivatives ^c		
Rx1	Wild-type recipient	31
DP1001	<i>ery-2</i>	31
DP1141	<i>thy-1</i>	J. E. Wagstaff (this laboratory)
DP1301	<i>cat ery-2</i>	BM6001 lysate × DP1001
DP1302	<i>cat tet ery-2</i>	BM6001 lysate × DP1001
DP1303	<i>cat tet ery-2</i> <i>str-1</i>	DP1617 DNA × DP1302
DP1304	<i>tet ery-2</i>	DP1617 DNA × DP1302
DP1320	<i>tet</i>	DP1302 lysate × Rx1
DP1321	<i>cat</i>	DP1302 lysate × Rx1
DP1325	<i>cat str-1 nov-1</i> <i>ery-2</i>	DP1617 DNA × DP1321
DP1617	<i>str-1 nov-1 ery-2</i>	Shoemaker
DP3200	Plasmid (<i>cat</i> <i>erm</i>)	See text

^a See text. Corresponding phenotypes are as follows: *cat*, Cm^r; *tet*, Tc^r; *thy-1*, Thy⁻; *str-1*, Str^r; *nov-1*, Nov^r; *ery-2*, Ery^r, resistant to erythromycin by alteration of a ribosomal protein; *erm*, Em^r, resistant to erythromycin by methylation of 23S rRNA (33). (We use two-letter symbols for phenotypes of known or suspected plasmid or transposon origin to distinguish them from those due to alleles of normal chromosomal genes [22].)

^b BM6001 and N77 were obtained from V. Hershfield (Duke University) as subcultures of lyophilized preparations received from the original workers.

^c All of the Rx strains listed except DP1617 are *hex*, lacking the marker-specific mismatch correction system that acts at the heteroduplex stage of integration (11, 31).

to a recipient Rx strain but at frequencies less than 1% of those expected for transformation by homologous point markers (Table 2, experiment 1). The level of *cat* transfer was close to that found by Dang-Van et al. (8). However, whereas they saw no transfer of *tet* from a DNA preparation from BM6001, we found Tc^r clones at a frequency eightfold lower than for chloramphenicol resistance. Two-thirds of the Tc^r clones were also Cm^r, but of the Cm^r clones only a few were

TABLE 2. *Transfer of chloramphenicol and tetracycline resistance determinants with an asymmetric linkage*

Expt	Donor ^a	Recipient ^b	Transformants per ml				Cotransfer ratio ^c	
			Ery ^r	Tc ^r	Cm ^r	Cm ^r Tc ^r	Cm ^r Tc ^r /Tc ^r	Cm ^r Tc ^r /Cm ^r
1	BM6001	DP1001	— ^d	1,520	12,000	— ^e	43/63	3/80
2	DP1302	Rx1	3.8×10^6	2,960	23,500	— ^e	166/214	16/183
3	DP1302	Rx1	3.0×10^6	2,660	22,000	1,690	0.64	0.08

^a A fresh lysate of donor cells was used at about 0.8 μ g of DNA per ml of recipient cells. DP1302 is one of the Cm^r Tc^r double transformants from experiment 1.

^b At the time of addition of DNA, these cultures contain about 4×10^7 cells per ml (2×10^7 colony-forming units per ml).

^c By replication of Cm^r and Tc^r colonies in experiments 1 and 2; by direct selection with both drugs in experiment 3.

^d BM6001 is not Ery^r and the recipient is. In a parallel transformation, this culture gave 2.3×10^6 Str^r colonies per ml. In later experiments using a *thy* recipient, the *thy*⁺ marker of BM6001 served as a reference, with comparable results.

^e Not determined directly.

also Tc^r. Prior addition of DNase prevented all transfer (data not shown), confirming that the process was transformation rather than transduction (27).

In these strains the marker discrimination system dependent on the *hex*⁺ gene is missing, and point markers show nearly identical high frequencies of transformation (11, 31). Therefore, the low frequencies of transfer of chloramphenicol and tetracycline resistance implied some unusual feature of the system. Of further significance was that a double transformant from the first experiment showed donor properties very similar to those of BM6001 (Table 2), suggesting that the unusual features were transferred largely intact to the transformants. Most further experiments were conducted on such derivatives, both singly and doubly transformed, as donors and as recipients.

The two classes of known genetic elements that might generate results of the kind seen are (i) plasmids that have to establish one or more new replicons in the recipient cell and (ii) lengthy insertions of nonhomologous DNA in a preexisting replicon of the normal genome. In the latter case there should be analogies to insertion of wild-type DNA into deletion recipients, a process that requires the donor strand inside the cell to be large enough both to span the deletion and to have sufficient flanking homology for efficient integration (1, 4, 12, 16; Shoemaker, unpublished data). In addition, there might be participation of active transposition elements, particularly in view of the likely ancestry of the *cat* and *tet* genes. Before presenting results that discriminate between plasmid and chromosomal loci, we will describe some features affecting the quantitative yield of Cm^r and Tc^r transformants.

Shear and homology effects. Table 3 summarizes representative results of experiments showing that two variables, prior shear of the donor lysate and increasing the degree of homology between donor and recipient strain, affect this system and interact with each other. For each combination of donor and recipient genotype, the effects of mild to moderate shear on the donor activities of *cat* and *tet* are shown normalized to the effect on the *ery-2* point marker (for which effects of DNA size on transformation efficiency have been reported extensively [6, 11, 20]). Transfer of *tet* was extremely sensitive to shear in all cases, whereas transfer of *cat* was less so. Blending the lysate in a Vortex mixer typically reduced the number of Tc^r transformants 10-fold and sometimes more, and repeated passage through a no. 27 needle essentially eliminated them. Extensive purification of the DNA by a Marmur procedure (18) also eliminated all transfer of *tet* (<2 Tc^r colonies per ml) but had little effect on *cat*, consistent with the prior report (8). Although the data shown for *tet* are representative of the median result of several experiments, between lysates the absolute values varied severalfold both before and after Vortex treatment. Also, *tet* donor activity in fresh lysates typically fell severalfold over a period of a few days at 4°C (data not shown), probably because of trace nuclease activity which was not sufficient to be detected by its effects on *cat* or *ery-2*. All of these results imply that in this system a very long DNA strand is needed for successful transfer of the *tet* gene. This could reflect either a large plasmid or a very long insertion.

Table 3 also shows a striking enhancement of transfer that was correlated with partial homology. The Tc^r recipient DP1320 was transformed

TABLE 3. *Effects of partial homology and shear on transfer of cat and tet^a*

Donor ^b	Shear	Recipient			
		Rx1 (Tc ^r / Ery ^r)	Rx1 (Cm ^r / Ery ^r)	DP1321 <i>cat</i> (Tc ^r / Ery ^r)	DP1320 <i>tet</i> (Cm ^r / Ery ^r)
DP1302 <i>cat tet</i>	None	0.001	0.0074	0.004	0.18
	Vortex	0.0001	0.0077	0.0002	0.07
	Needle	~10 ⁻⁶	0.0047	~10 ⁻⁶	0.04
DP1301 <i>cat</i>	None		0.15		0.20
	Vortex		0.11		0.17
	Needle		0.04		0.06
DP1304 <i>tet</i>	None	0.001		0.004	
	Vortex	0.0001		0.0002	
	Needle	~10 ⁻⁶		— ^c	

^a Entries are relative numbers of the indicated classes of transformants observed (compare Table 2).

^b Donors were gently prepared fresh lysates sheared as indicated. All carried *ery-2* plus *cat* or *tet* as indicated.

^c —, Not done.

to Cm^r by the *cat tet* donor 30-fold more readily than was Rx1. The presence of *cat* in the recipient (DP1321) increased receptivity for *tet* two- to fourfold from either a *cat tet* or a *tet* donor lysate. Also, singly marked *cat* strains (DP1301 and others not shown) were 20-fold-better donors of *cat* to Rx1 than were *cat tet* donors. The singly marked *tet* strain did not, however, transfer *tet* to Rx1 significantly better than did the *cat tet* donor.

In contrast to *tet*, transfer of *cat* from *cat tet* donors was not sensitive to Vortex treatment, possibly even rising slightly, and was only slightly more sensitive to needle shear than was *ery-2*. (The latter data are the means for six experiments.) However, in all of those crosses in which the transformation efficiency for *cat* was increased, it was reproducibly more sensitive to both levels of shear. Therefore, whatever process had operated to enhance the efficiency was itself sensitive to the length of the donor strand.

Replacement of recipient marker. In the cross DP1301 to DP1320, 27 of 440 Cm^r transformants tested were Tc^r; in the cross DP1304 to DP1321, 28 of 385 Tc^r transformants were Cm^r. Thus, in 6 to 7% of the transformants, the donor marker either replaced or displaced the recipient marker.

If, as in the case of tetracycline resistance, the low efficiency of transfer of chloramphenicol resistance from a *cat tet* donor reflected a need for entry of a rather long strand, then its transfer should have been reduced more strongly by

shear than it was (Table 3). Instead, the data show that needle-sheared preparations containing a distribution of fragments with a median size of 5 to 7 megadaltons (6) were capable of transformation to chloramphenicol resistance nearly as efficiently as were preparations containing much larger DNA particles. Because the relative frequency of Cm^r and Ery^r transformants was independent of donor DNA concentration (data not shown), the low efficiency even for large donor molecules could not be ascribed to a need for independent transformations by two markers.

The data presented so far could be consistent with either plasmid or chromosomal insertion hypotheses. The experiments that follow show that the latter is correct, but do not in themselves explain the low efficiency for transfer of *cat* from *cat tet* donors.

Cosedimentation of *cat* with chromosomal markers. Figure 1a shows sedimentation velocity runs on a lysate of BM6001 that had been either blended with a Vortex mixer or needle sheared. The activities in the unsheared lysate sedimented more rapidly (data not shown), but even in it little *tet* activity was recovered from the fractions. In all cases, *cat* cosedimented with the chromosomal marker (*thy*⁺), and, of most significance, its velocity distribution was shifted by the shear treatment. Comparable results were found for lysates of DP1301 and DP1302. In the needle-sheared lysate, the decreased Cm^r/Thy⁺ ratios in the slower fractions were consistent with the shear sensitivity of *cat*, suggesting that its transfer required a segment of a few kilobases in size, somewhat larger than that needed for efficient transfer of a point marker (6). Cosedimentation data have been used previously to demonstrate integration of an ampicillin resistance determinant into the *Haemophilus influenzae* chromosome (3).

In contrast, the *erm* donor activity on the plasmid in DP3200 sedimented very differently from the chromosomal *thy*⁺ (Fig. 1b). Similar complex profiles for plasmid transformation in *Bacillus subtilis* reflect multimeric forms of much higher donor activity than the monomer (5), a result which illustrates the complexity of the transfection process in gram-positive organisms (7, 9, 10, 26). It is specifically relevant here in that plasmid activity profiles do not follow that of chromosomal DNA as does *cat* in BM6001 and its derivatives. In other experiments the plasmid *cat* marker in DP3200 behaved like *erm*, and we have yet to find a transformant in which the plasmid markers have failed to cotransfer.

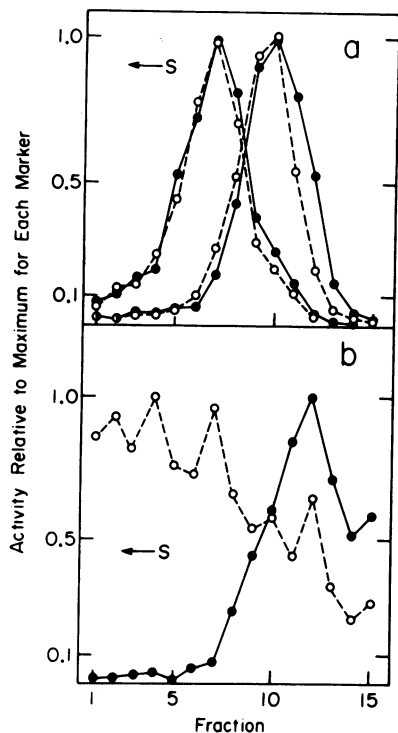


FIG. 1. Comparison of sedimentation of donor activities of *cat* from BM6001 and *erm* from DP3200 with that of the chromosomal *thy+* marker. (a) BM6001, lysate blended in a Vortex mixer (left pair of curves) or needle-sheared lysate (right pair of curves) (symbols: ○, *cat*; ●, *thy+*). (b) DP3200, blended in a Vortex mixer (symbols: ○, *erm*; ●, *thy+*). Samples (0.1 ml) of fresh lysates were layered on 5 to 20% gradients of sucrose in 0.15 M NaCl–0.015 M sodium citrate–0.05 M Tris-hydrochloride, pH 7.6, and centrifuged for 3 h at 33,000 rpm (a) or 25,000 rpm (b). Fractions were collected and assayed for transformation on DP1141 (*thy-1*).

Dye-buoyant density. Distributions of transforming activity in EtBr-CsCl equilibrium gradients are shown in Fig. 2 for strains DP1301 and DP3200. The latter shows the majority of the plasmid *erm* activity well resolved from the chromosomal marker, at a position consistent with that of closed circular DNA (28). Some plasmid transforming activity also appeared to be associated with open circle or linear DNA. For the DP1301 donor, however, all of the *cat* activity cobanded with the chromosomal marker at the position expected for linear DNA; similar results were found for DP1303. Although by this criterion *cat* activity might have resided on open circle or linear forms of a plasmid, it should then have shown a unique velocity distribution in experiments such as those shown in Fig. 1, which it did not. Therefore, the *cat* activity in both *cat*

and *cat tet* donors was carried on linear DNA fragments of essentially the same length distribution as that of the total chromosomal DNA.

Linkage of *cat* to a chromosomal marker.

In confirmation of the physical results above, genetic results show that *cat* is linked to a chromosomal gene (Table 4). It cotransferred with *nov-1* significantly more often than expected for random double events or than observed for its cotransfer with other markers, when the donor lysate was not sheared, whereas gentle shear abolished the excess for both *cat nov* and the known *str ery* linkage (29). The shear reduced *Cm^r Nov^r/Cm^r* from 3.5 to 0.7% and *Str^r Ery^r/Str^r* from 6.6 to 0.7%, where the lower values are near those expected for independent events calculated as described previously (25). To see the nonrandom cotransfer clearly, it was necessary to use a donor concentration such that DNA was limiting. With the usual 25-fold-more-concentrated lysates, the independent double events increased as expected and obscured the linkage (data not shown). Selection artifacts were ruled out both by this result and by that seen after shear.

Is *tet* also in the chromosome? In the above experiments the recovery of *tet* activity was too low to demonstrate directly its behavior, and therefore the argument that *tet* is in the chro-

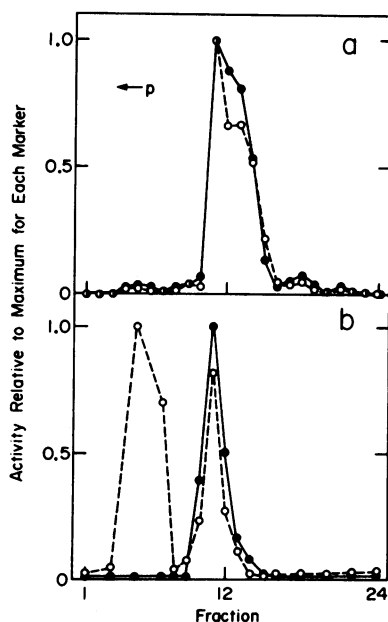


FIG. 2. Distribution of *cat*, *erm*, and chromosomal marker transforming activities in EtBr-CsCl equilibrium gradients (see text). (a) Lysate of DP1301 (symbols: ○, *cat*; ●, *ery-2*). (b) Lysate of DP3200 (symbols: ○, *erm*; ●, *thy+*).

TABLE 4. Linkage of *cat* to chromosomal *nov* marker^a

Resistances scored ^b	Transformants per ml by following treatment of donor lysates:	
	No shear	Vortex
Str	330,000	350,000
Ery	320,000	320,000
Nov	300,000	300,000
Cm	51,000	20,000
Str Ery	21,000	2,310
Str Nov	3,980	1,880
Ery Nov	2,720	1,330
Cm Str	487	167
Cm Ery	410	138
Cm Nov	1,810	139

^a Two samples, each containing 4×10^7 DP1325 cells, were lysed in 0.05 ml, diluted with 0.5 ml of saline-citrate, and heated for 5 min at 60°C to kill any remaining cells. One sample was blended in a Vortex mixer for 2 min, and each was then exposed to 5 ml of competent Rx1 cells for 15 min at 37°C before addition of 50 μ g of DNase I. The DNA concentration during exposure was limiting (near 0.03 μ g/ml). After another 20 min of incubation at 37°C, the cells were diluted and plated. After 60 min of additional incubation at 37°C, an 8-ml agar layer was added, followed by a second 8-ml layer containing drug(s).

^b Str, Streptomycin; Ery, erythromycin; Nov, novobiocin; Cm, chloramphenicol.

mosome depends on its cotransfer with *cat* (Table 2). However, the yield of double transformants was much smaller than the number of cells which should have taken up two point markers independently (near 10^5 /ml in these experiments). If the survival of *tet* in the recipient were in some way strongly promoted by the simultaneous presence of *cat*, and to a lesser extent the converse, then the observed cotransfer might not require the two markers to be physically linked in the donor. Two results conflict with this hypothesis, as follows. The transfer of *tet* was only slightly enhanced by the presence of *cat* in the recipient (Table 3), and as shown in Table 5, a mixture of *cat* and *tet* donor lysates gave double transformants only at the frequency expected for independent events. We conclude that *tet* is physically linked to *cat* in the *cat tet* strains and is therefore also integrated in the chromosome.

Clinical isolate from Niigata. Strain N77 (19) has behaved very similarly to the Paris strain BM6001 in the following respects (data not shown). (i) N77 and derivatives of it generated results very similar to those shown in Tables 2 and 3 with respect to transfer frequencies, asymmetric cotransfer of *cat* and *tet*, and shear sensitivities. (ii) As donors, the *cat* and *tet* derivatives of N77 showed enhanced transfers to the Paris *cat* and *tet* derivatives at levels similar to

those shown in Table 3. (iii) The *cat* marker from N77 showed linkage to *nov-1* with results essentially identical to those shown in Table 4. We conclude that there are at most small differences in the behavior of the *cat* and *tet* regions of N77 and BM6001.

Gel electrophoresis. All attempts to detect unique forms of DNA by electrophoresis of cleared or uncleared lysates of BM6001, DP1301, DP1303, and N77 have given negative results, yielding only smooth distributions of heterogeneous chromosomal DNA (reference 32 and experiments not shown). In control experiments, two forms of the 20-megadalton plasmid in DP3200 were readily detected in cleared lysates, even when made from mixtures of DP3200 and BM6001 cells, and the same was true for the 2-megadalton pDP1.

DISCUSSION

Recent Cm^r and Tc^r isolates of pneumococcus have a chloramphenicol acetyltransferase (8, 19) and other properties, including "curing" by EtBr (8) and low frequency of transfer of their chloramphenicol and tetracycline resistance, that suggested that the resistance genes might be carried on plasmids. However, the following three lines of evidence have demonstrated that the *cat* determinant is on the chromosome of strain BM6001 and its laboratory derivatives: (i) the cosedimentation of *cat* with chromosomal markers over a wide range of velocities (Fig. 1), (ii) its cobanding with chromosomal markers in dye-CsCl buoyancy gradients (Fig. 2), and (iii) its nonrandom cotransfer with *nov-1* (Table 4). Because *tet* is linked to *cat* (Tables 2 and 5), it also is in the chromosome. It is important to emphasize that the failure to detect plasmid DNA in these strains, although consistent with the positive evidence on the location of *cat* and *tet*, is not the basis for the conclusion.

Given chromosomal insertion and the probable plasmid ancestry of the *cat* and *tet* determi-

TABLE 5. Linkage of *tet* to *cat*

Donor ^a	Transformants per ml		
	Cm ^r	Tc ^r	Cm ^r Tc ^r
DP1302	6.0×10^3	305	197
Mixture of DP1301 and DP1304	2.2×10^5	197	2.5 ^b

^a Lysates of the indicated strains were used with Rx1 recipients at a concentration near 4×10^7 cells per ml.

^b Three colonies in 1.2 ml were scored on six plates. If *cat* independently transformed Tc^r transformants at the same frequency as it did all other cells, the expected number was 1 to 2. Conversely, the Tc^r transformants observed here were 10^{-5} of the total population and of the Cm^r transformants.

nants, the clear possibility exists that they have transposed into the chromosome and may still be part of transposable elements. They may be like the "pseudoplasmids" recently shown to be transposons in *Staphylococcus aureus* (23, 24). However, we cannot draw this conclusion in the absence of evidence for active transposition, and the evidence so far does not demand it. Instead, we show below that much of the behavior of this system is that expected for transformation of regions of nonhomology by legitimate pairing processes and requires no assumptions about transposition. Although a class of low-efficiency crossover events must be postulated to account for some of the results, these may simply reflect limited homology.

Organization of the *cat tet* region. Given that *cat* and *tet* are linked on the chromosome, the data strongly imply that they are arranged as shown in Fig. 3, where *cat* is part of a nonhomologous insertion and *tet* is included in a much larger insertion. The insertions are joined in *cat tet* strains by a region H of unknown extent in which crossovers can occur to the region H' of the wild-type recipient but are rare compared with those expected for lengthy homology between H and H'. We shall return to the possible nature of H and H' after discussing the evidence for the arrangement shown in Fig. 3.

The evidence for nonhomology is the extreme shear sensitivity of the transfer of *tet*, which implies that its successful integration requires entry of a very long strand, either to bridge the region of nonhomology or to complete a transposon. (Donor DNA after entry is single stranded in this system [17].) The effective unit of transfer appears to be the total region, of which *tet* itself presumably is a small part. The fact that transfer of *cat* also requires longer

DNA strands than does that of a point mutation (Table 3 and Fig. 1) implies that it also has extensive nonhomology to the normal genome. The lack of homology shows that pneumococcus acquired these genes from one or more foreign donors and is consistent with their residence on plasmids at unknown stages of their ancestry. Preliminary considerations of the shear sensitivity of the transfers and of prior measurements of distributions of intracellular donor strand lengths (20, 21) suggest that the *cat* region from abc to H may be 4 to 8 kilobases long and that the *tet* region, H to def, probably exceeds 30 kb. A large part of the low efficiency of *tet* almost certainly reflects the fact that very few strands this long get into the cell, even for very large donor DNA, because they are cut nonspecifically at the cell surface into strands of median length near 7 kilobases before entry (21).

If *cat* and *tet* are inserted at the same sites in the singly marked as in the doubly marked strains (Fig. 3), the strong enhancement of transfer of *cat* from a *cat tet* donor to a *tet* recipient and from a *cat* donor to Rx1, as compared with that from *cat tet* to Rx1, is that expected for the much reduced region of nonhomology and the greater length of flanking homology available for pairing. As the latter is reduced by shear, the efficiency falls, but for a given shear treatment it remains greater than that for *cat* from the *cat tet* donor (Table 3).

Asymmetric cotransfer and the efficiency of integration of *cat*. Although crossovers clearly can occur in the region between *cat* and *tet*, two results, the asymmetry of their cotransfer and the low but shear-resistant efficiency of integrating *cat* from *cat tet* donors, are not expected if the efficiency of crossover in this region is as high as that using lengthy homologous regions such as those at abc and def. After entry, few of the donor strands from a *cat tet* strain span the entire region from abc to def, but many span the *cat* region and still do not integrate *cat*. This suggests that *cat* most commonly integrates, when it does so at all, by using the abc homology to initiate the process and a much lower efficiency process in the region H to complete it. On this model, removal of the *tet* region is irrelevant or even helpful to the probability of the second crossover in H, and *cat* transfer is less sensitive to shear of the *cat tet* donor (Table 3). Alternatively, if the *cat* region is a transposon, it might integrate by a mechanism unrelated to the neighboring homology. However, the donor DNA is single stranded, efficient pairing at abc is an expected event, and all evidence to date implies that transfer among these strains is site specific.

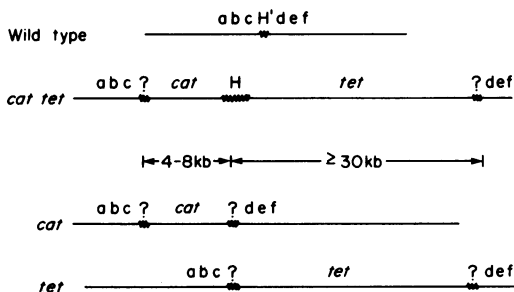


FIG. 3. Organization of the *cat* and *tet* regions. H and H' represent regions of unknown extent and character between which crossovers can occur but do so rarely compared with those in the homologous regions at abc and def. Regions indicated by ? may represent part or all of H' or H (see text).

On the asymmetric cotransfer of *tet* and *cat*, there should be fewer strands carrying the full region *abc cat H tet def* than those with only *H tet def*, yet at least twice as many successful integrations arise from the former as from the latter (Tables 2 and 5). This also would result if crossover in *H* were inefficient compared with that in the fully homologous regions at *abc* or *def*. This suggests that integration of *tet* requires a long strand extending from *def* through *tet* itself, is greatly enhanced if the strand extends to *abc*, but can occur by crossover in *H*. Conversely, the number of strands extending from *abc* through *cat* to *H* is much larger than those extending to *def*, and the result is that the low-efficiency event in *H* yields most *cat* transfers without *tet*.

On the nature of *H* and *H'*. The above analysis shows that most of the transformation properties of the *cat* and *tet* determinants are consistent with the organization suggested, provided that we add the new feature of low-efficiency crossovers between the regions *H* and *H'*. The mechanism of these events, however, is unknown at this time. Although an active role for insertion sequences or a site-specific process such as in the lambda *att-int* system is a possibility as noted earlier, most of the results can readily be explained by passive models. These include short complete or lengthy partial homologies between *H* and *H'*, repetitious sequences, and totally illegitimate random crossovers. If *H'* exists between *c* and *d* on the wild-type chromosome as suggested, then it or a part of it may be to the left of *cat* and to the right of *tet* as indicated in Fig. 3. However, *H'* could be simply the *cd* junction or even a sequence that is deleted during integration.

It is in crosses between singly marked *cat* and *tet* strains that the results suggest complexities in the *H-H'* interaction. If *cat* and *tet* are each flanked by *abc* and *def* as shown in Fig. 3, double crossovers in these regions could lead to the replacement of one by the other (Fig. 4). The fact that this occurred in 6 to 7% of the transfers of the donor marker supports the interpretation that in these strains *cat* and *tet* had integrated at or near the same position. However, integration of the donor marker was enhanced 30-fold for *cat* and 2- to 4-fold for *tet* (Table 3), even though the apparent nonhomology between donor and recipient was as great as that between *cat tet* and wild type, and almost all of it resulted from addition rather than replacement. This suggests that these *cat* and *tet* strains share some feature of *H* that promotes crossover in this region and is not present in the cross of *cat tet* to wild type. It may simply be that the

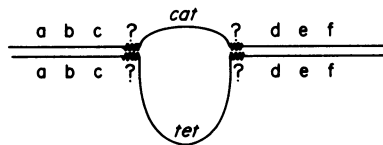


FIG. 4. Pairing for replacement of *tet* by *cat* or vice versa (see text).

particular strains tested so far have substantial overlaps in the segment of *H* integrated, although in testing other *cat* and *tet* derivatives from both BM6001 and N77 we have yet to find a combination that does not show enhancement (data not shown).

Transposition? The possibility that transposition contributes some of the transfers in this system cannot be excluded. However, for it to account for all of the results, it would have to be true (i) that legitimate pairing of the kind we have discussed would not occur with high efficiency in lengthy regions of homology, in conflict with experience in pneumococcus and *B. subtilis*, (ii) that *cat* and *tet* transpose predominantly to sites adjacent to one another by a mechanism that is independent of the surrounding homology but sensitive to shear of the donor DNA, and either (iii) that the wild-type chromosome contains an insertion sequence that acts on the single strand of donor DNA or (iv) that an insertion sequence on the single-stranded donor can be transcribed and promote integration of the donor. (i) seems highly unlikely, and we defer judgment on (ii) and (iv) until some evidence is obtained. This system is clearly different from most of those in which transposition has been studied so far.

On the origins of *Cm'* *Tc'* strains of pneumococcus. Whatever the mechanism that allows crossover in the region between *cat* and *tet*, the results suggest that these genes are arranged the same in BM6001 and the *cat tet* derivatives studied so far and that transformation of wild-type strains by them is aided and dominated by the surrounding homology rather than by exclusive use of an illegitimate insertion mechanism. However, the question remains open as to how the genes were first acquired by the progenitor of BM6001. They may have entered as plasmid DNA or as segments of foreign chromosomes in which they were already integrated. The fact that *Tc'* strains appeared earlier and are still more common than *Cm'* *Tc'* strains (8) suggests that *cat* and *tet* entered separately. A number of serotypes are represented in the Paris collection (8), and it may be that independent events led to *Tc'* strains and later one or more of them acquired *cat* from a second source. Alternatively, *tet* may have integrated only once from a foreign

source and then been donated to other pneumococcal strains. In any event, the question arises as to where a foreign DNA carrying *cat* or *tet* would integrate if not aided by homology. To date, the only relevant information is as described above, that strain N77 from Japan and its derivatives appear to have these genes integrated in similar large insertions at or near the same site as in BM6001 and its derivatives. However, it cannot be excluded that these strains, even though isolated in different parts of the world, received their *cat* and *tet* regions from a common ancestor, nor do the data so far exclude small differences between the sites of insertion into the chromosome.

More generally, the present studies suggest a mechanism for evolution that we do not believe has been emphasized before in this particular context. Namely, given a pathway for physical transfer of DNA, there can be an autocatalytic accumulation of genes from one or more heterologous sources in a process whose result mimics that of plasmid-carried resistance transfer, without there being plasmids in the species of concern. Acquisition of one segment of heterologous DNA strongly increased chromosomal receptivity for further DNA from the same or similar donors (Table 3). It is probable that another foreign DNA homologous to any part of the long *tet* region would have an increased chance of integration into a Tc^r strain of pneumococcus. Duncan et al. (9) have made a similar point with respect to plasmid-carried foreign DNA in *B. subtilis*. Further studies may show whether such mechanisms have operated in generating the multiply drug-resistant South African strains (2, 15), which also lack a detectable plasmid (L. W. Mayer, and V. B. Ploscowe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, D26, p. 36).

ACKNOWLEDGMENTS

We thank V. Hershfield for strains and for discussion of the work and the manuscript and V. L. Lee for preparation of the competent cells and assistance with assays.

This work was supported by contract EY-76S-05-3941 from the Department of Energy and by Public Health Service grant GM21887 from The National Institutes of Health to W.R.G. M.D.S. is a genetics trainee under Public Health Service grant GM02007 from the National Institute of General Medical Sciences.

ADDENDUM

A paper has appeared giving further results on the transduction and transformation of two linked transposons in *S. aureus* (24), including asymmetric co-transfer and 8% recombinational displacement of one by the other in Rec⁺ cells. From studies in Rec⁻ recipients, the authors conclude that active transposition plays a part in the transfer of one of the inserted elements but not the other.

LITERATURE CITED

1. Adams, A. 1972. Transformation and transduction of a large deletion mutation in *Bacillus subtilis*. *Mol. Gen. Genet.* 118:311-322.
2. Applebaum, P. C., H. J. Koornhof, M. Jacobs, R. Robins-Browne, M. Isaacson, J. Gilliland, and R. Austrian. 1977. Multiple antibiotic-resistance of pneumococci-South Africa. *Morbidity Mortal. Weekly Rep.* 26:285-286.
3. Bendler, J. W., III. 1976. Physical size of the donor locus and transmission of *Haemophilus influenzae* ampicillin resistance genes by deoxyribonucleic acid-mediated transformation. *J. Bacteriol.* 125:197-204.
4. Bernheimer, H. P., and I. E. Wermundsen. 1972. Homology in capsular transformation reactions in pneumococcus. *Mol. Gen. Genet.* 116:68-83.
5. Canosi, U., G. Morelli, and T. A. Trautner. 1978. The relationship between molecular structure and transformation efficiency of some *S. aureus* plasmids isolated from *B. subtilis*. *Mol. Gen. Genet.* 166:259-267.
6. Cato, A., and W. R. Guild. 1968. Transformation and DNA size. I. Activity of fragments of defined size and a fit to a random double crossover model. *J. Mol. Biol.* 37:157-178.
7. Contente, S., and D. Dubnau. 1979. Characterization of plasmid transformation in *Bacillus subtilis*: kinetic properties and the effect of DNA conformation. *Mol. Gen. Genet.* 187:251-258.
8. Dang-Van, A., G. Tiraby, J. F. Acar, W. V. Shaw, and D. H. Bouanchaud. 1978. Chloramphenicol resistance in *Streptococcus pneumoniae*: enzymatic acetylation and possible plasmid linkage. *Antimicrob. Agents Chemother.* 13:577-583.
9. Duncan, C. H., G. A. Wilson, and F. E. Young. 1978. Mechanism of integrating foreign DNA during transformation of *Bacillus subtilis*. *Proc. Natl. Acad. Sci. U.S.A.* 75:3664-3668.
10. Gryczan, T. J., S. Contente, and D. Dubnau. 1978. Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. *J. Bacteriol.* 134:318-329.
11. Guild, W. R., and N. B. Shoemaker. 1976. Mismatch correction in pneumococcal transformation: donor length and *hex*-dependent marker efficiency. *J. Bacteriol.* 125:125-135.
12. Harris-Warrick, R. M., and J. Lederberg. 1978. Interspecies transformation in *Bacillus*: mechanism of heterologous intergenote transformation. *J. Bacteriol.* 133:1246-1253.
13. Hershfield, V. 1979. Plasmids mediating multiple drug resistance in group B streptococcus: transferability and molecular properties. *Plasmid* 2:137-149.
14. Horodniceanu, T., D. H. Bouanchaud, G. Bieth, and Y. A. Chabbert. 1976. R plasmids in *Streptococcus agalactiae* (group B). *Antimicrob. Agents Chemother.* 10:795-801.
15. Jacobs, M. R., H. J. Koornhof, R. M. Robins-Browne, C. M. Stevenson, Z. A. Vermaak, I. Freiman, G. B. Miller, M. A. Witcomb, M. Isaacson, J. I. Ward, and R. Austrian. 1978. Emergence of multiply resistant pneumococci. *N. Engl. J. Med.* 299:735-740.
16. Lacks, S. 1966. Integration efficiency and genetic recombination in pneumococcal transformation. *Genetics* 53:207-235.
17. Lacks, S., B. Greenberg, and K. Carlson. 1967. Fate of donor DNA in pneumococcal transformation. *J. Mol. Biol.* 29:327-347.
18. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. Mol. Biol.* 3:208-218.
19. Miyamura, S., H. Ochiai, Y. Nitahara, Y. Nakagawa, and M. Terao. 1977. Resistance mechanism of chlor-

- amphenicol in *Streptococcus haemolyticus*, *Streptococcus pneumoniae*, and *Streptococcus faecalis*. Microbiol. Immunol. (Tokyo) 21:69-76.
20. Morrison, D. A., and W. R. Guild. 1972. Transformation and deoxyribonucleic acid size: extent of degradation on entry varies with size of donor. J. Bacteriol. 112:1157-1168.
 21. Morrison, D. A., and W. R. Guild. 1973. Breakage prior to entry of donor DNA in pneumococcus transformation. Biochim. Biophys. Acta 299:545-556.
 22. Novick, R. P., R. C. Clowes, S. N. Cohen, R. Curtiss III, N. Datta, and S. Falkow. 1976. Uniform nomenclature for bacterial plasmids: a proposal. Bacteriol. Rev. 40:168-189.
 23. Novick, R. P., I. Edelman, M. D. Schwesinger, A. D. Gruss, E. C. Swanson, and P. A. Pattee. 1979. Genetic translocation in *Staphylococcus aureus*. Proc. Natl. Acad. Sci. U.S.A. 76:400-404.
 24. Phillips, S., and R. P. Novick. 1979. Tn554—a site-specific repressor-controlled transposon in *Staphylococcus aureus*. Nature (London) 278:476-478.
 25. Porter, R. D., and W. R. Guild. 1969. Number of transformable units per cell in *Diplococcus pneumoniae*. J. Bacteriol. 97:1033-1035.
 26. Porter, R. D., and W. R. Guild. 1978. Transfection in pneumococcus: single-strand intermediates in the formation of infective centers. J. Virol. 25:60-72.
 27. Porter, R. D., N. B. Shoemaker, G. Rampe, and W. R. Guild. 1979. Bacteriophage-associated gene transfer in pneumococcus: transduction or pseudotransduction? J. Bacteriol. 137:556-567.
 28. Radloff, R., W. Bauer, and J. Vinograd. 1967. A dye-buoyant density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc. Natl. Acad. Sci. U.S.A. 57:1514-1521.
 29. Ravin, A. W., and K.-C. Chen. 1967. Heterospecific transformation of pneumococcus and streptococcus. III. Reduction of linkage. Genetics 57:851-864.
 30. Shoemaker, N. B., and W. R. Guild. 1972. Kinetics of integration of transforming DNA in pneumococcus. Proc. Natl. Acad. Sci. U.S.A. 69:3331-3335.
 31. Shoemaker, N. B., and W. R. Guild. 1974. Destruction of low efficiency markers is a slow process occurring at a heteroduplex stage of transformation. Mol. Gen. Genet. 128:283-290.
 32. Smith, M. D., and W. R. Guild. 1979. A plasmid in *Streptococcus pneumoniae*. J. Bacteriol. 137:735-739.
 33. Weisblum, B., S. B. Holder, and S. M. Halling. 1979. Deoxyribonucleic acid sequence common to staphylococcal and streptococcal plasmids which specify erythromycin resistance. J. Bacteriol. 138:990-998.